λInCh

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Contents

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STRAIN LIST

SM551 E. coli K12 F-, (λ)-, Δlac(MS265), mel-1, nalA2, supF58 (=suIII, tyrT58)
This strain was derived from Mike Malamy's QL which was derived from the classical strain Y-Mel (Bachman 1996 in E. coli and Salmonella. Neidhardt et al eds p2460-2488, Chart 1.)

DHB6501=SM551

JOE59 SM551 (λInCh1). This is λInCh for pBR.

DHB6521=JOE59

DHB6500 SM551 (λInCh2). This is λInCh for pUC.

Both lambda vectors are cI857, Sam7. They contain a plasmid DNA and chromosomal DNA cloned in BsiWI + SacI digested parental lambda. Both confer kanamycin resistance. They have the Tn903 KanR allele from pUC4K.

λInCh Manual

Dana Boyd
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Introduction.

λInCh is a tool which can be used to simplify the process of transferring an expressed gene from a plasmid onto the E. coli chromosome. It is a bacteriophage lambda vector. The name InCh stands for Into the Chromosome. Transfer of the genetic material onto the chromosome is accomplished in three in vivo steps involving homologous recombination and site specific recombination. In the first step, homology between sequences on a typical pBR322 derived plasmid and sequences on λInCh permit a double recombination, which confers ampicillin resistance to the phage and results in pickup of the plasmid insert. In the second step the ampicillin resistance gene in the phage and the linked plasmid insert are put onto the chromosome of E. coli by site specific recombination of the phage into the l att site. In the third step most of the λ DNA is deleted by a second homologous recombination event. To make this step possible the phage carries a fragment of chromosomal DNA from one side of the att site, near att DNA, in a position such that most of the lambda DNA can be looped out and deleted by a single recombination event.

This is a draft version of the user manual. The first section is a simple linear protocol that should work. Subsequent sections discuss variations and give a little more detail on the mechanisms. There is some repetition.

A simple protocol.

This is an example of how to proceed. Other approaches might be more appropriate for your combination of plasmid and host strain.

First, you must grow a λInCh lysate. The strain Joe59 or DHB6521 is lysogenic for λInCh1. λInCh2 can be used in the same way.

a. Streak Joe59 or DHB6521 on a broth plate and incubate overnight at 30°C
b. Pick a single colony and grow a 5ml overnight culture in broth at 30°C.
c. Dilute 100X in 5ml broth with 2mM MgSO4.
d. Incubate at 42C for 15 minutes.
e. Incubate at 37°C with good aeration. Lysis should be apparent after about 1 hour.
f. Add 1/100th volume of chloroform and vortex.
g. Continue incubation with aeration at 37°C for 15 min.
h. Spin 10,000 RPM 10 min at 4°C.
i. Decant the supernatant into a sterile tube with 1/100th volume of chloroform and vortex. This is the primary λInCh1 lysate. Store it at 4°C.

Second, you can titer the lysate on a permissive strain.

a. Grow a 5ml overnight culture of SM551 or DHB6501 from a single colony in broth at 37°C.
b. Dilute 100X in 5ml broth with 2mM MgSO4 and 0.2% maltose.
c. Incubate at 37 °C with aeration until OD600 is 0.2.
d. Put the culture in ice. This is the plating culture.
e. Make serial 10X dilutions of the lysate in 5ml broth with 2mM MgSO4. Make dilutions of 10-6 to 10-12.
f. Mix 0.1 ml of each lysate dilution with 0.1ml of plating culture.
g. Incubate 5 minutes at 37°C.
h. Add 3.3 ml of melted 0.7% top agar at 47°C to the mixture of lysate and plating culture. Vortex and immediately pour onto a broth agar plate containing 2mM MgSO4 and 0.2% maltose. Distribute the melted agar uniformly before it hardens.
i. Incubate at 37°C overnight.
j. Determine the phage titer by counting plaques on a plate with 30 to 300 colonies.

Third, make a lysogen in your plasmid containing strain. Be sure that your plasmid confers ampicillin resistance and is capable of pickup by λInCh1. (The Homology Detector applications, InCh for pBR and InCh for pUC, which run on Macintosh computers are useful for determining whether your plasmid insert can be picked up.)

a. Grow an overnight culture from a single colony of your strain in broth with 2mM MgSO4 0.2% maltose and 0.2mg/ml of ampicillin to select for maintenance of your plasmid.
b. Mix 0.1 ml of overnight with 108 λInCh1 and incubate at 30°C for 15 minutes. Dilute to 1 ml in broth with 2mM MgSO4 and incubate with aeration for 1 hour at 30°C.
c. Plate serial dilutions on media with 0.2mg/ml ampicillin and 0.04mg/ml kanamycin and incubate overnight at 30°C.
d. Pick and purify by successive restreaking several colonies from the highest dilution plate that has any colonies. These should be λInCh lysogens of your strain.
e. Test these strains by streaking on a pair of plates. Incubate one at 42°C and the other at 30°C overnight. Lysogens should have no or few colonies at 42°C but should grow normally at 30°C. Choose a your primary lysogen for the next step.

Fourth, grow a lambda inch lysate from your primary lysogen. This is similar to the first step above except that lysis must be induced with chloroform (unless your strain is a supF strain, which is permissive for the growth of λInCh1). During growth of the phage a double recombination event can result in transfer of ampicillin resistance and your plasmid insert onto the phage DNA. This is a reasonably common event even though the amount of homology is not great because recombination frequencies are high during lambda growth.

a. Pick a single colony of your lysogen and grow a 5ml overnight culture in broth at 30°C selecting for maintenance of your plasmid with ampicillin.
b. Dilute 100X in 5ml broth with 2mM MgSO4.
c. Incubate at 42C for 15 minutes.
d. Incubate at 37°C with good aeration for 3 hours.
e. Add 1/100th volume of chloroform and vortex.
f. Continue incubation with aeration at 37°C for 15 min. Lysis may or may not be apparent.
g. Spin 10,000 RPM 10 min at 4°C.
h. Decant the supernatant into a sterile tube with 1/100th volume of chloroform and vortex. This is the low frequency transducing (LFT) lysate. Most of the phage particles should carry the KanR allele of λInCh1 but some should have recombined with your plasmid and carry the ampR gene (and your insert) instead.

Fifth, make an ampicillin resistant secondary lysogen. This step involves insertion of the recombinant lambda onto the chromosome by site specific recombination, the second of the three recombination events mentioned above. This is an efficient process.

a. Grow an overnight culture of DHB6501 in 5ml broth with 2mM MgSO4 and 0.2%maltose.
b. Mix 0.1ml serial 10-fold dilutions of the LFT lysate with 0.1 ml aliquots of the culture.
c. Incubate 15 min at 30 °C, dilute to 1 ml in broth with 2mM MgSO4 and incubate 1 hour at 30°C.
d. Spread 0.1 ml of each culture on broth plates with 0.025mg/ml ampicillin. Concentrate the cells in the culture with the highest number of phage by centrifugation and plate on the same medium. The low concentration of ampicillin used in this step is appropriate for use with pBAD and pDHB60 derived plasmids. The Ampicillin resistance gene on these plasmids confers only low level resistance when in single copy.
e. Incubate overnight at 30°C
f. Pick colonies from the lowest dilution plate that has colonies. These should be lysogenic for λInCh1 with your insert recombined into the phage.
g. Purify several colonies on broth agar plates without antibiotics and test for AmpR, KanR, and growth at 42°C and 30°C. Chose your secondary lysogen from among the colonies with the correct phenotype, AmpR, KanS and TS.
Note that it is possible to transduce the plasmid in this step, presumably as a cointegrate which is resolved upon lysogenization. Use of high concentrations opf ampicillin selects for this.

Sixth, grow a high frequency transducing (HFT) lysate. Make and titer a lysate from the strain obtained in step 5 above, the secondary lysogen, using the procedures of steps 1 or 4 and 2 above.

Seventh, select lysogens in your target strain(s) using the HFT lysate and the procedures of step 5 above but with your strain(s) as recipient and the HFT lysate as the phage stock. These tertiary lysogens are the precursors of the final cured strains you will construct. This step again involves site specific recombination at the att site. It actually repeats the second of the three recombination events that are involved in the construction.

Eighth, cure the tertiary lysogens to stabilize the chromosomal construct. This step involves homology dependent recombination between the chromosomal region just before the att site and a region in the prophage. This is the third recombination involved in the construction. This deletion results in loss of all functional lambda genes and almost all the lambda DNA. Cells with the deletion are no longer temperature sensitive so they can grow at 42°C.

a. Grow overnight cultures in broth at 30°C
b. Plate dilutions on broth plates and incubate overnight at 42°C
c. Pick well isolated colonies from the plates with the fewest cells plated.
d. Purify by streaking on broth plates and incubating at room temperature.
e. Test the cured strains for antibiotic resistance and temperature sensitivity as in step 5. The cured strains should be resistant to .025mg/ml ampicillin and no longer temperature sensitive. You may want to test them for correctness using PCR. Suggestions for doing this will be given elsewhere.

Ninth, transducing the ampicillin resistance. The ampicillin resistance allele should be 100% linked to your plasmid insert at the att site on the chromosome in the cured strains. You can transduce it to other strains with P1 selecting resistance to .025mg/ml ampicillin. To do this either cure the secondary lysogen obtained in step 5 using the method outlined in step 8 or use one of the cured strains obtained in step 8. Grow P1 on the cured strain and use the lysate to transduce ampicillin resistance.

Note that all of the steps above may be modified and many may be omitted. For example if you omit step 2, titering the primary lysate, you can use a series of dilutions of the phage stock in the third step and pick colonies from the lowest dilution that gives resistant colonies. This can be thought of as titering transducing particles instead of the phage. Similarly it is not necessary to make a primary lysogen as outlined in step 3. It is likely that a phage stock could be grown directly on your plasmid containing strain by infection a MOI=5 or so. This approach may speed things up.

A major alternative is to introduce your plasmid into Joe59 or DHB6521 and make the LFT lysate from that. Your strain may not be appropriate for growth of lambda for one reason or another. It might be lambda resistant, or it might be a lambda lysogen already. Transformation of your plasmid into Joe59 or DHB6521 is actually a safer first step unless your plasmid might cause trouble in strains containing it.

1. Make DHB6521 competent and transform your plasmid into it.
2. Induce and prepare an LFT lysate. Expect lysis about 1 hour after induction. 3. 3. Continue from step 5 above.

Some steps (or their equivalent) are important. It may be tempting to use the LFT lysate to construct strains but this is not correct. The recombinant phage in the LFT lysate are not clonally derived. The ampicillin resistant phage in the LFT lysates arise from many independent recombination events. If your plasmid differs from lambda InCh in the regions of shared homology, this could be result in differences among the ampicillin resistant recombinants in the LFT lysate. An alternative to making a secondary lysogen is to identify recombinant plaques from the LFT lysate by plating on an indicator lawn and grow an HFT lysate from a single recombinant plaque.

It is also important to include the HFT lysate step to avoid transduction of the plasmid. It is not safe to conclude that the primary AmpR lysogen has no plasmid. Transduction of the plasmid as a homogenotized cointegrate and resolution of the cointegrate on lysogenization is common. (There is selection against the persistence of the cointegrate in the chromosome.) HFT lysates made from such a primary lysogen may also contain a minority of plasmid transducing particles. For some purposes in is advisable to make a second HFT lysate using a lysogen obtained from the first HFT lysate. The idea is to be sure of leaving the plasmid behind.

General λInCh Methods

λInCh1 carries a C-terminal fragment of the pBR322 bla gene and a piece of pBR322 DNA that is between the tet gene and the origin of replication. (I pronounce λInCh as "lambda inch.") Both fragments are present in many pBR322 derived cloning vectors. During growth of λInCh on strains containing such plasmids ampicillin resistance and the cloned insert DNA can be transferred to the phage by a double recombination event. This works even in recA strains because the lambda vector has its own highly active recombination system. In four (or 5) efficient, in vivo steps this DNA can be transferred to the chromosome in single copy at the att site. The final strain is not a lambda lysogen, so is quite stable. λInCh1 is also called λInCh for pBR, λDL1.2, λDHB6521 and λJOE59.

The two fragments of pBR322 are the PstI, at 3607, to DraI, at 3249 "'bla" piece and the PvuII, at 2064, to AflIII, at 2473, "near ori" piece. If your plasmid has these DNA fragments with your DNA inserted between them, in the position of the pBR322 tet gene, and is ampicillin resistant you can use this method. λInCh1 works with a variety of pBR322 derived, ampicillin resistant expression systems including pDHB Tac promoter plasmids, pBAD plasmids, pKK Tac promoter plasmids, pTrc plasmids, some new green fluorescent protein plasmids and a large number of other plasmids.

λInCh2 is similar and will work with pUC plasmids, pUC18 and 19 for example, and pUC like plasmids, some pGEM plasmids for example. The relevant pUC18 fragments are a near ori lacZ alpha piece, AflIII at 2560 (across 0/2686) to EcoRI at 230, and a 'bla piece from ScaI at 1189 to DraI at 1784. The multi cloning site is between EcoRI at 230 and HinDIII at 281. λInCh2 is also called λInCh for pUC and λDHB6500.

Neither of these vectors is recommended for pET T7 expression system plasmids or pBLUESCRIPT plasmids. New versions of λInCh should be constructed for use with these systems.

A. Growth of lysates on an AmpR plasmid containing strain.

1. Requirements for growth of lysates

There are several ways to make λInCh lysates. The phage are cI857 Sam. Such cI857 Sam lysogens can be constructed and induced in most normal lab strains even though the essential S gene has an amber mutation in it and the cI repressor has the 857 mutation. The cI857 allele is temperature (heat) sensitive. It is inactivated at 42°C resulting in induction of lysogens but forms lysogens normally at 30°C. (Between 34 and 37°C It has intermediate behavior.) A suppressor host strain can be used to grow Sam lysates but it is not necessary to suppress the S amber mutation to get lysates. S is required for normal lysis and plaque formation but complete phage particles are formed after infection or induction in non suppressing hosts. Since the host does not lyse at the normal time the intracellular titer continues to increase until it is as much as 10 to 100X higher than in a normal lysis. These phage can be liberated with chloroform (optionally after concentration of the cells.) Such lysates have a few S^+ phage in them as the S

allele can revert. Since these phage can grow lytically they may become a problem. This can be avoided during selection of lysogens by plating at room temperature or at 20°C because λ cannot inject its DNA at room temperature and cannot enter the lytic cycle at 20°C.

There are a few other requirements for working with lambda. Lambda requires the outer membrane protein LamB for adsorption, so in any infection the host must express LamB. It is ordinarily not necessary to induce with maltose (*lamB* is in the *malB* operon) as the uninduced level is sufficient, but it is customary to do so. Lambda particles require Mg++ for stability. Phage particles with more than the wildtype amount of DNA packaged less efficiently and are less stable. There is not a sharp cutoff in the amount of DNA that can be packaged but particles between 5 and 20% bigger than wildtype are increasingly short lived.

2. Growing l lysates.

To grow a lysate you can follow the instructions in Miller, J. 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. p320. or Arber et al Experimental methods for use with lambda, in Lambda II Hendrix et al eds Cold Spring Harbor Laboratory, Cold Spring Harbor, New York pp433-466.

a. Growth on a non-suppressor host.

i. by infection. To grow lytically in a non suppressing host containing a plasmid, infect an early log culture at 37°C in broth with 1 -10mM Mg and 0.1 -0.2% maltose at a multiplicity of infection (MOI) of 5 to 10. Incubate 10 min without shaking and then 3 hours with aeration. Concentrating the cells 10 fold during the 10 minute adsorption step improves the efficiency and synchrony of infection. After 3 hours add about 1% by volume of chloroform and continue aeration for 5 to 10 minutes. Concentrating the cells before this step can give higher titers but is not necessary unless a DNA preparation is to be made. Lysis should be evident. Clarify the sup by centrifugation and store the lysate, the sup, with a drop of chloroform in the dark at 4°C.

ii. by lysogenization and induction. To construct primary lysogens use a stationary phase broth culture of a strain with an AmpR plasmid made 1-10 mM in $MgSO_4$ and infected with lambda InCh. The most efficient way to do this is to concentrate the cells 10 fold, add the phage at room temperature and incubate for 15 minutes, dilute 10 fold shift to 30°C. This procedure allows adsorption of the phage (this is a slow process at any temperature) at the low temperature and simultaneous injection of all adsorbed phage after the temperature shift. After incubation at 30°C for 1 hour the cells are plated on media containing both ampicillin and kanamycin and incubated at 30°C or below. The methods outlined above will give the most efficient lysogenization, but the selection is so strong that log phase cells and low multiplicities of infection should work fine provided Mg++ is present and the temperature is 30°C. Isolated colonies should be restreaked for purification maintaining only ampicillin selection and tested for resistance to both antibiotics.

To make a lysate from a lysogen, grow an overnight culture of the ampicillin and kanamycin resistant strain obtained above in broth at 30°C. Dilute and grow to

early log phase at 30°C. Shift to 42 to 45°C for 15-20 minutes. Shift to 37°C for 3 hours with good aeration. Spin out the cells. Resuspend in 1/20th volume of broth with 1mM Mg SO_4 (or in SM buffer, 0.12MNaCl. 1mM $MgSO_4$, 20mM Tris HCl pH 7.5, 0.01% gelatin). Add about 1-2% of the current volume of chloroform and shake at 30°C for about 10 minutes until lysis is evident. If the lysate is viscous add 0.001 volume of 1mg/ml DNase I and incubate until it is no longer viscous, a few minutes. Spin out the debris. The supernatant is the lysate.

b. Growth on an amber suppressor host.

The Sam mutation can be suppressed by supF. A variety of strains have this suppressor. The derivatives of Ymel called QL, JF50, SM551 and DB644 and DHB68 are unusually healthy for K12 strains. SM551, in Susan Michaelis's strain collection (in the Beckwith lab), is F- del lac(MS265), mel, nalA, supF58 (=suIII). LE392 is a commonly available supF host and other supF strains are listed in the NEBL catalog.

With a suppressing strain either plate or liquid lysates can be prepared by standard methods. The methods are similar to those used for P1 except the plates or broth should contain 1mM $MgSO_4$ instead of $CaCl_2$.

B. Selection of ampicillin resistant lysogens.

The lysates obtained above by growth on plasmid containing strains should have a minority population of phage that will confer ampicillin resistance as lysogens. These arise by recombination and will have the DNA between 'bla and near ori from the plasmid in the prophage on the chromosome. The majority population should confer kanamycin resistance and another minority population may confer resistance to both antibiotics. To select ampicillin resistance infect a suitable, rec+ host with the phage as described above for selection of primary lysogens and plate on 25 micrograms per ml of ampicillin at 30°C. Use a stationary phase culture with 1mM $MgSO_4$ added. The strong selection should make it possible to use 30°C throughout. Plating at room temperature or 20°C would eliminate any problems with lytic phage but should not be necessary. It may be useful to try several different dilutions of the lysate to so that lysogens are derived from singly infected cells. The lysogens should be purified at 30°C without selection and tested for ampicillin resistance and temperature sensitivity at 42°C.

C. Growth of high frequency transducing lysates.

The strains obtained above should be induced to make pure lysates in which all the phage carry the AmpR allele.

D. Selection of temperature independent recombinants.

The strains obtained in step B above (or derived from lysates made in step C) are temperature sensitive because induction of the prophage at 42°C kills the host. The presence of an 800bp DNA fragment from the chromosome between gal and att, "near att" DNA, in the prophage results in occasional (about 1/10,000) loss of most of the phage DNA by recombination. These recombinants are ampicillin resistant but no longer temperature sensitive. This step is probably dependent of the host recA system, so rec+ strains should be used for this step.

Grow an overnight culture of the ampicillin resistant lysogen obtained above at 30°C and plate on medium with 25 micrograms per ml of ampicillin at 42°C. Plate various dilutions and pick from the lowest dilution with colonies. The S+ revertant phage are definitely a problem at this stage as they can grow lytically on the colonies which are no longer immune to lambda. This appears as nibbled colonies on the selection plate. Purify on plates without antibiotic at room temperature to purify away from these phage. Test for ampicillin resistance and temperature sensitivity.

The temperature independent strains obtained in this way should have bla, your DNA and the near ori DNA inserted in the chromosome at the att site. Almost no lambda genes, nothing significant, should remain. They should not be immune to lambda. The strain will have a hybrid att site near your insert so should be capable of lambda lysogen formation. The ampicillin resistance (to 25 micrograms per ml) should be transducable by P1 and 100% linked to your DNA. The inserted DNA should be as stable as the gal bio region in any other strain unless you have inserted DNA with homology to some other part of the chromosome. In the event that homology with another part of the chromosome is an issue recA derivatives can be constructed.

How to tell if a λInCh vector will work with your plasmid.

If your plasmid is related to pBR322 or the pUC plasmids you can probably use λInCh1 or λInCh2 to put the insert onto the chromosome in single copy. You should determine if the correct sequences are actually in your plasmid before you try. For lInCh1, if your insert is cloned between the DraI, at 3249 to PstI, at 3607 "'bla" segment and the PvuII, at 2064, to AflIII, at 2473 "near ori" segment of pBR322 (or similar plasmid) and a double recombination event will transfer a functional bla gene or other antibiotic resistance to the phage, you can probably use lInCh1. For pUC18 the relevant fragments are a near ori lacZ alpha piece, AflIII at 2560 (across 0/2686) to EcoRI at 230, and a 'bla piece from ScaI at 1189 to DraI at 1784.

a. Homology Detectors

Homology Detectors are Macintosh specific, HyperCard based, shareware application software that simplify the process of deciding if you have homology. You simply paste your sequence into a window and the application draws a map of the homology for you. The instruction for use are contained within the applications.

b. Blast

It should be possible to do a Blast search in the internet using your plasmid to find its homology to pBR322 or pUC18 but I have not yet tried this.

c. DNA Strider

You can also use DNA Strider's Matrix-DNA Matrix command (or similar DNA analysis software) to examine the homology between your plasmid and the relevant part of the Lambda Inch vectors. DNA Strider is also Macintosh specific. It is not shareware and not available with λInCh, but is in wide use in labs where plasmids and Macintosh computers are used. To use the Matrix-DNA Matrix command the monitor must be set to 256 colors. You will need a Strider file of your plasmid and

the files "lambda inch insert" and "lambda inch insert.RC". Use the command Edit-Unlock Editing on your plasmid file. Next use Find-Jump To set the cursor somewhere in the replicative origin region of your plasmid. Now use Conv-Set Origin to set the 0 position of your plasmid to the point in the replicative origin region. The plasmid must have Conv-Circular checked in the Conv menu for this to work smoothly. Finally save your plasmid file with a new name.

Now put your new plasmid file in the front window and click somewhere in the DNA sequence without selecting anything. Next put "lambda inch insert" or "lambda inch insert.RC" in the front window and click somewhere in the sequence without selecting anything. Next use the Matrix-Stringency and Matrix-Window commands to set the window and stringency both to 7 or more. Finally Use the Matrix-DNA matrix command to compare the DNA's. Be sure to compare your plasmid to both "lambda inch insert" and "lambda inch insert.RC". Diagonal lines indicate homology. If you have two diagonal lines, one in near ori region and the other in the 'bla IG region you can pick up whatever is between them on the lambda and if you can select for it, it should work. Try this with something known to work to see what it should look like before trying your plasmid.

If the above sounds complicated, it is; the Homology Detectors are much simpler.

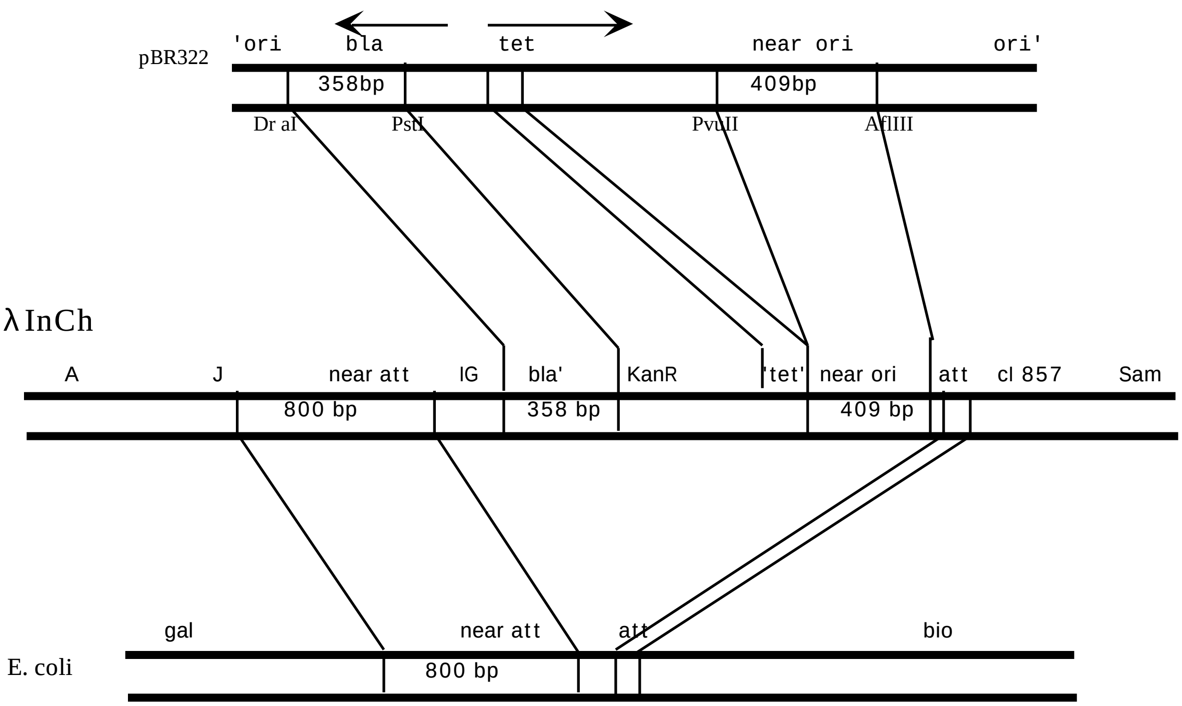
How much DNA will fit in lambda? An untested method.

Phage particles with more than the wildtype amount of DNA are less stable and more dependent on magnesium for stability. There is not a sharp cutoff in the amount of DNA that can be packaged but particles between 5 and 20% bigger than wildtype are increasingly short lived. Also, particles with significantly less than the wildtype amount of DNA, less than 75% or so, are not efficiently packaged.

There are 48505bp in lambda. The nominal allowed size limits are 75% to 105% of lambda so 36 to 51 kb are the nominal limits. The λInCh for pBR, InCh1, insert is 3750 bp. It replaces a BsiWI-SacI fragment which is 6555 bp long. So it is 2805 bp shorter than lambda, 45700 bp. But 613-2077 of the insert is KanR which will be replaced. so only 2286 is added to the final recombinant by the vector lambda making it 44236 plus whatever recombines in. So, since the nominal limit is 51kb, there is room for 6.8 kb from the plasmid between the homology regions. λInCh2 is similar but not identical.

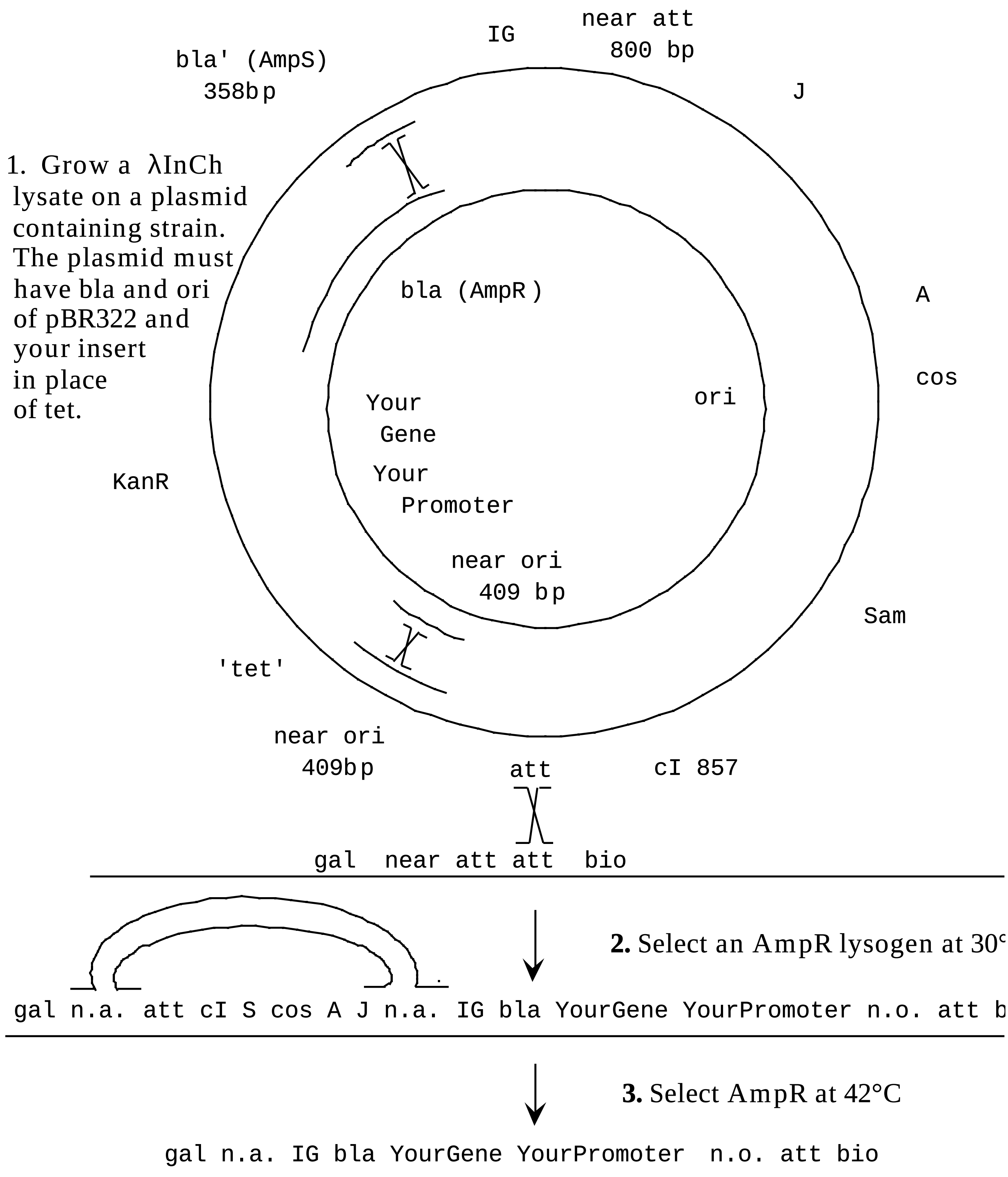
Note that the nominal limit is not an absolute limit. Larger phage are unstable, but they do exist. I have not tried to use large λInCh derivatives but I have packaged cosmids that are larger than lambda . To minimize the time during which the unstable particles must exist it should be possible to use a host like SM551 which is supF and streptomycin sensitive. Put the plasmid into Joe59, a λInCh1 lysogen of SM551. Preinduce by incubation at 42°C for 15 minutes, mix with a non suppressing streptomycin resistant recipient, incubate for 2 hours at 30 °C, and plate on medium containing both ampicillin and streptomycin. Since the phage particles containing the large cosmid can immediately infect the recipient on lysis, the problem of instability should be minimized. I do not know what the practical size limit is.

λInCh1 Homologies



λInCh Crossovers

1. Grow a λInCh lysate on a plasmid containing strain. The plasmid must have bla and ori of pBR322 and your insert in place of tet.

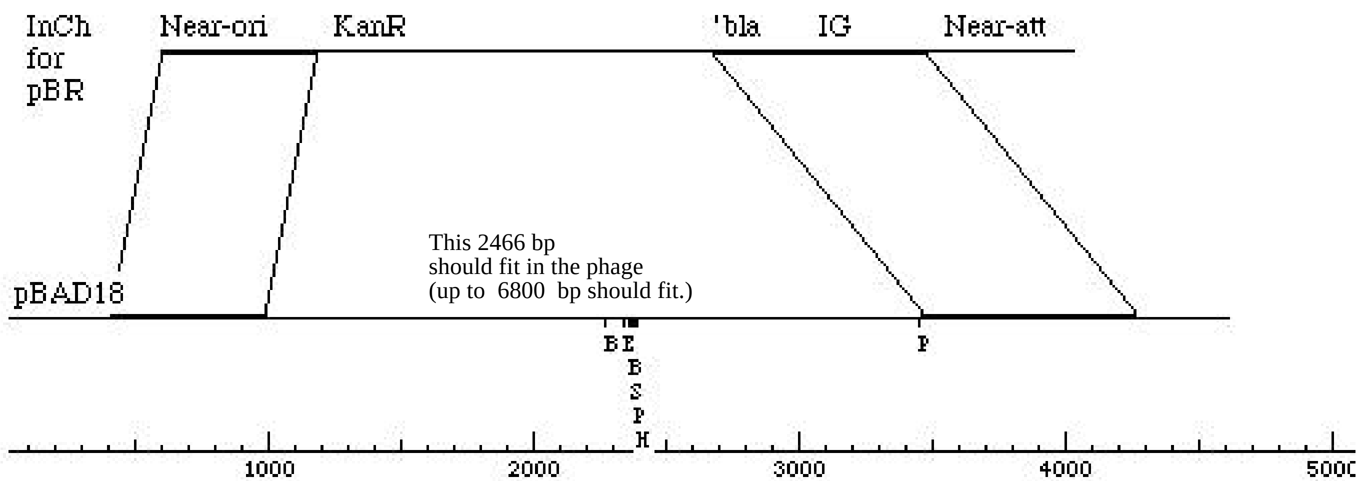


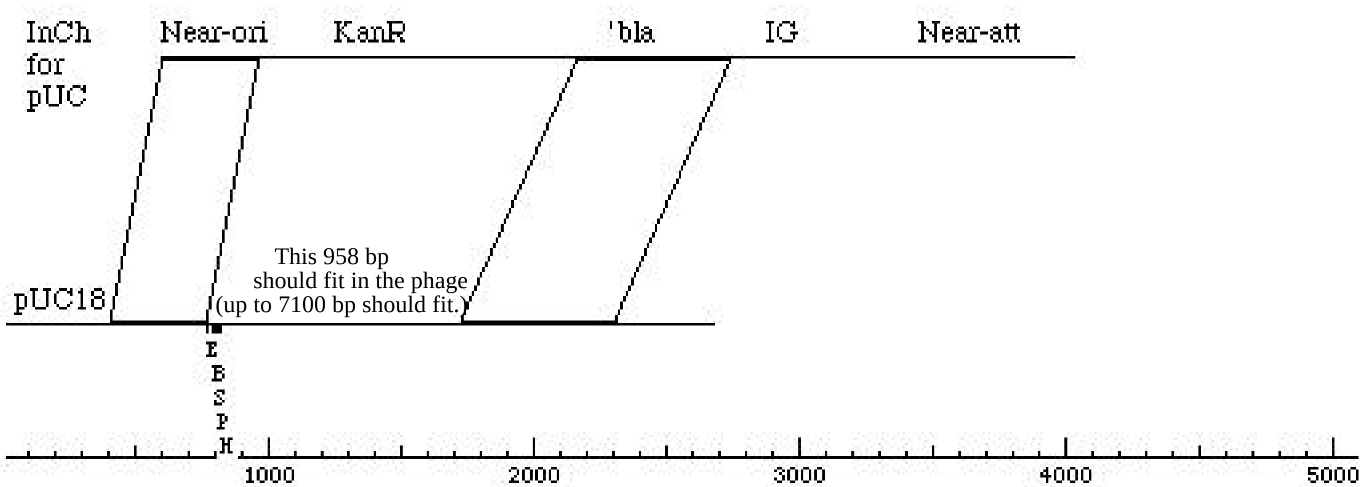
2. Select an AmpR lysogen at 30°

3. Select AmpR at 42°C

λInCh Homology Detectors

Homology Detectors make maps of the homology between plasmids and λInCh





OUTLINE

Lambda INCH, a simple E. coli plasmid/chromosome shuttle system.

Dana Boyd, David Weiss. Joe Chen and Jon Beckwith

Abstract

We describe a simple system for reversible stable integration of plasmid borne genes into the E. coli chromosome. Most ordinary E. coli strains and variety of pBR322 derived ampicillin resistant plasmids can be used. A single genetic element, a lambda phage is the only specialized vector required. The resultant strains have a single copy of the plasmid fragment inserted stably at the lambda attachment site on the chromosome and are not lysogens.

Introduction

1. Why

Because of their high copy number pBR32 derived plasmids are easy to work with. High level expression from such multi-copy plasmids is sometimes desirable but for physiological measurements low level expression from a single chromosomal copy is often required. We have devised a simple system for integrating the expression system form pTac (like pDHB32()) pTrc (), pBAD () and green fluorescent protein (). The same system should work for a wide variety of pBR322 related plasmid vectors including ...

2. How

The system employs bacteriophage lambda as a vector and is similar to a method described previously(). A lambda phage with plasmid DNA flanking the insert can recombine with the plasmid. On the lambda is part of the bla gene and some DNA from near the origin of pBR322. Since these regions flank the insert region, a double recombination completing the phage borne bla gene results in incorporation of the DNA of interest into the phage. This can then insert into the chromosome at the lambda attachment site.

Although lambda lysogens are stable enough for most purposes, they are less stable than ordinary chromosomal loci. In addition multiple lysogens which are readily formed when antibiotic resistance is used to select lysogens pose a special problem. For this reason we have cloned a fragment of the E. coli chromosome flanking the attachment site into lambda InCh. This makes it possible to delete almost all of the lambda DNA leaving only a single stable copy of the insert on the chromosome.

Finally, we describe a simple system for recovering the inserted DNA from the chromosome by recombining it back onto a high copy plasmid so it can easily be characterized.

3. Generality

This method should be generally useful with a wide variety of ampicillin resistant pBR322 derived plasmids that are in common use. It does not require special strains of E. coli. Insertion into the chromosome requires only the phage vector lambda InCh. Versions usable with pUC plasmids and with kanamycin resistance plasmids are available. Recovery of inserted DNA is possible with the use of additional plasmid and a helper M13 or f1 phage.

Methods

1. How to make a lambda lysogen from the strain with a plasmid in it.
a. Non-suppressing
To construct primary lysogens a stationary phase broth culture of a strain with an ampR plasmid made 5mM in MgSO4 and infected with lambda INCH. Lysogens are formed more efficiently at multiplicities of infection above 1 but the ratio of phage to cells is not important if only a few lysogen are required and there is a strong selection. After incubation at 30°C for 1 hour the cells are plated on media containing both ampicillin and kanamycin.
b. suppressing
With a suppressing strain either plate or liquid lysates can be prepared.
2. How to grow a lambda lysate from the strain
a. non suppressing
Pick a single colony from the selection plate and streak once on amp medium to purify it. After overnight growth at 30°C pick a single colony to broth containing ampicillin and incubate at 30°C overnight. (At this time test for resistance to kanamycin and for temperature sensitivity by streaking at 42°C.) Dilute the culture into fresh broth and grow at 30°C to log phase. Shift the culture to 42°C for 15 minutes and then incubate for 2.5 to 3 hours at 37°C with aeration. Spin out the cells and resuspend in 1/100th to equal volume of broth with 5mM MgSO4. Add chloroform and continue aeration at 37°C until lysis is obvious. Spin out the debris and the chloroform, the supernatant is the lysate. If it is viscous add DNAse I (about 0.1 microgram/ml) and incubate until viscosity is reduced. The resulting lysate contains mostly kanR phage along with some ampR phage and some phage that have both resistances.
b. suppressor
With a suppressor strain either plate or liquid lysates can be prepared by standard methods.

3. How to select the secondary lysogens.
Secondary lysogens are selected in the same way as the primary lysogens (above) except that the host strains is initially amp sensitive and ampicillin resistance is selected. Amp resistant kanamycin resistant temperature sensitive lysogens should all have the desired insertion the phage at the lambda attachment site.
4. How to make the deletions.
Plate the amp resistant temperature sensitive lysogenson medium with ampicillin at 42°C. The efficiency of plating should be ...X lower at 42°C than at 30°C.
5. How to get the insert back off the chromosome onto the plasmid.
Put KanR plasmid with IG region in the AmpR striain. Grow M13 and select AmrR transductants.

Results

1. Pickup of the plasmid DNA onto lambda.
 a. How to tell if your plasmid will work.

b. How to do it.
c. Some data with various plasmids

2. Titer of lysates suppressing and non suppressing
3. Making the lysaogens.
   a. Data on frequency
4. Making the deletions.
   a. Data on frequency
5. Confirmation by PCR

Discussion

1. Ease of use and efficiency issues
2. Stability of deleted vs. ordinary lambda lysogens.
3. Ideas for externsion
   a. Using other plasmids
   b. Using other att sites.

Growth of att+ cI857 Sam Lambda InCh vectors

All solutions should contain Mg++. I use 5mM MgSO4 in broth such as LB or in Tris buffered saline pH7.5. There are better methods.

I. Induction of lysogens.

A. SM551 and other supF strains.

Grow to mid-log phase in broth with Mg++ (5mM) at 30°C.
Shift to 42°C for 15 min.
Incubate at 37°C with aeration until lysis, about 1 hour.
Add chloroform, vortex and spin.
The supernatant is the lysate.

B. Non supressor strains

Grow to early log phase at 30°C or dilute a fresh overnight 50 fold in broth with Mg++ (5mM).
Incubate at 42°C 15 min
Incubate at 37°C with aeration 3 to 5 hours.
(Optional, concentrate 10 to 100 X by centrifugation and resuspension in something with 5mM Mg++)
Add 1% by volume chloroform and continue incubation for 10 min.. The culture should lyse. (If you have concentrated the cells the sup may be viscous. If so add a small amount of DNaseI and incubate until viscosity is eliminated. Try 0.1 micrograms per ml DNaseI, the right amount is the amount that takes about 5 to 10 minutes to eliminate viscosity)
Spin out the debris. The sup is the lysate.

2. Titration of lysates on SM551.

Make serial dilutions in Mg++ containing broth.
Mix 0.1 ml aliquots with 0.1 ml of early log SM551 grown in broth + Mg++ and maltose.
Incubate 10 min at 30 or 37°C.
Mix with 3ml of soft ager and pour. I add Mg++ and maltose to the soft agar.
Incubate at 30 or 37°C.

Poorer medium, like tryptone broth or lambda broth will give bigger plaques
Non supressing strains induced as above and not concentrated give 2X10 to the 10th power plaque forming units/ per ml of lysate.

3. Growth of plate lysates on SM551 and other supressor strains.

Titrate the lysate as above.
Use a barely confluent plate to make a plate lysate. Titers are haghest after about 6 hours of incubation, but overnight incubation is OK for most Lambda InCh purposes.

4. Infection in liquid culture of both supressor and non supressor strains.

Mix phage and cells at a MOI (multiplicity of infection) of 5 to10 phage per cell. More does mnot seem too be very deleterious.

Incubate at 37 or 42°C for 10 minutes.

Dilute and incubate at 37°C until lysis, at 1 hour if it is SM551 or after 3 to 5 hours incubation and addition of chloroform if it is a non supressor strain. Proceed as with induced cultures above.

5. Other growth methods.

Sam phage may also be grown on supressor strains by other methods. For example, infection of a low OD culture at a low MOI can be used to give two or more rounds of growth as in the PDS method used for Lambda Charon vectors.

Confirmation of Structure.

After deletion of lambda by recombination during selection of temperature independence, the structure at the insertion site can be confirmed by PCR. We use 4 oligonucleotides for this . The chromosome with the inserted lambda can be represented as :

```
gal_______near-att___att_int_CI__S__A__J__near-att__IG__bla__INSERT__no__att__bio
```

and after deletion:

```
gal_______near-att __IG__bla__INSERT__no__att__bio
```

The primers are shown below:

Non-lysogenic parent

```
gal_____near-att____att________bio
gal_f->          <-att_r
```

Lysogen

```
gal________near-att____att_____int_CI_S_A_J___near-att _____IG__bla__INSERT__no__att__bio
gal_f->            <- att_r <-int_r                     <-IG_r
```

Cured by deletion

```
gal________near-att ______IG_bla__INSERT__no__att__bio
gal_f->                 <-IG_r
```

		Product Size (bp.)		
		Non-lysogenic parent	Lysogen	Cured
Primer1	Primer2			
gal_f->	<-att_r	977	977	*
gal_f->	<-int_r	-	1128	-
gal_f->	<-IG_r	-	-	1148

* Sites too far apart to give product.
- No site for second primer.

The sequences of the primers used are:

```
gal_f->   5'-CTTGCTGAGTACGTGAGTTC-3'
<-att_r   5'-AAGCAGGCTTCAACGGATTC–3'
<-int_R   5'-GGACACCATGGCATCACAGT–3'
<-IG_r    5'-ACGTTGGAGTCCACGTTCTT–3'
```

For a .05 ml reaction we use eacn primer at .001mM and .002ml of culture with the following temperature cycles:

1 cycle	95 degrees C	1min
25 cycles	95 degrees C	1min
	45 degrees C	1min
	72 degrees C	2min